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Blocking hedgehog signaling after ablation of the dorsal neural tube allows regeneration of the cardiac neural crest and rescue of outflow tract septation

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ABSTRACT

Cardiac neural crest cells (CNCC) migrate into the caudal pharynx and arterial pole of the heart to form the outflow septum. Ablation of the CNCC results in arterial pole malalignment and failure of outflow septation, resulting in a common trunk overriding the right ventricle. Unlike preotic cranial crest, the postotic CNCC do not normally regenerate. We applied the hedgehog signaling inhibitor, cyclopamine (Cyc), to chick embryos after CNCC ablation and found normal heart development at day 9 suggesting that the CNCC population was reconstituted. We ablated the CNCC, and labeled the remaining neural tube with DiI/CSRE and applied cyclopamine. Cells migrated from the neural tube in the CNCC-ablated, cyclopamine-treated embryos but not in untreated CNCC-ablated embryos. The newly generated cells followed the CNCC migration pathways, expressed neural crest markers and supported normal heart development. Finally, we tested whether reducing hedgehog signaling caused redeployment of the dorsal–ventral axis of the injured neural tube, allowing generation of new neural crest-like cells. The dorsal neural tube marker, Pax7, was maintained 12 h after CNCC ablation with Cyc treatment but not in the CNCC-ablated alone. This disruption of dorsal–ventral neural patterning permits a new wave of migratory cardiac neural crest-like cells.

Introduction

Neural crest cells originate from the dorsal neural tube and are divided broadly into cranial and trunk regions based on their ability to give rise to ectomesenchyme; only the cranial neural crest has this capacity (Horstadius, 1950). Cardiac neural crest is a subdivision of the cranial crest and originates from the mid-otic placode through somite 3, corresponding to rhombomeres 6–8 (Kirby et al., 1985). Cardiac neural crest represents a transitional region between the cranial and trunk crest because it shares some properties common to both regions. It generates ectomesenchyme like cranial crest and it lacks the ability for regeneration like trunk crest (Suzuki and Kirby, 1997).

Cardiac neural crest cells (CNCC) migrate from the neural tube into caudal pharyngeal arches (arches 3–6) where they modulate FGF8 signaling and support pharyngeal arch artery development (Hutson et al., 2006; Waldo et al., 1996). A subset of the CNCC migrates into the distal cardiac outflow tract to form the aorticopulmonary septum. Ablation of premigratory CNCC cause the cells to be absent from the caudal pharynx and from the outflow tract. This results malalignment of the outflow tract because of excessive FGF8 signaling in the caudal pharynx, and common arterial trunk due to the failure of aorticopulmonary septation (Kirby et al., 1983). Excessive FGF8 signaling interferes with addition of the final myocardium to the outflow tract and disrupts the normal cardiac looping process which leads to malalignment of the arterial trunks with respect to the ventricles.

Scherson et al. (1993) ablated the neural crest in the caudal midbrain and pre-otic hindbrain and found that cells at the same axial level emigrated from the remaining neural tube along normal pathways to reconstitute a population of neural crest cells which gave rise to all the normal neural crest derivatives. These investigators concluded that neural tube cells normally destined to form CNS derivatives can adjust their prospective fates to form PNS and other neural crest derivatives until 4.5–6 h after normal onset of emigration from the neural tube. A second study by Suzuki and Kirby (1997) showed that the postotic neural tube lacks this ability to reconstitute a population of neural crest cells. It is not currently known why this regulative ability is limited to the neural tube in the caudal midbrain and rostral hindbrain.

Signaling by the secreted morphogen, Sonic hedgehog (Shh), plays key role in many aspects of vertebrate development including dorsal-ventral patterning of the neural tube (McMahon et al., 2003; Nishi et al., 2009). Shh is expressed in the notocord and floorplate of the neural tube and forms a gradient along the DV axis in the neural tube to specify progenitors in the ventral neural tube in a concentration-dependent manner (Briscoe and Ericson, 1999; Marti et al., 1995; Roelink et al., 1995). Exposure of dorsal neural tube to Shh causes adjacent neural tube cells to differentiate into ventral neural tube cell fates (van Straaten et al., 1985). Treatment of neural

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tubes prior to neural tube closure to Shh can prevent neural crest migration from the dorsal neural tube *in vivo* and *in vitro* (Selleck et al., 1998; Testaz et al., 2001). Neural crest cells arise from dorsal neural tube precursors in response to BMP signaling initiated by the dorsal epidermis (Dickinson et al., 1995). Interestingly, Shh treatment does not affect dorsal *Bmp* expression (Selleck et al., 1998). These results point to a possible role for ventral Shh signaling in regulating neural crest cell migration.

In this study we disrupted Shh signaling by applying Cyc to chick embryos after neural crest-ablation (HH10). To our surprise 53% of the hearts from neural crest-ablated, Cyc-treated embryos had normally aligned, septated outflow tracts as opposed to 5% normal hearts from ablated embryos not treated with Cyc. We hypothesized that reducing hedgehog signaling by Cyc resulted in repatterning of the dorsal–ventral axis in the injured neural tube, thus allowing the formation of new neural crest cells. To test this we ablated the CNCC, and labeled the remaining neural tube with fluorescent tracer and applied Cyc. Examination of the embryos showed that after 24 h treatment, cells emigrated from the neural tube in the CNCC-ablated (CNCC-A), Cyc-treated embryos but not in the CNCC-A, PBS-treated. Expression of Pax7, a dorsal neural tube marker, was maintained 12 h after neural crest ablation in the neural tubes of CNCC-A, Cyc-treated embryos. The emigrating cells followed normal CNCC migration pathways, expressed the neural crest markers and formed the aorticopulmonary septum. This suggests that Cyc treatment disrupts the dorsal–ventral patterning of the injured neural tube permitting a new wave of cardiac neural crest-like cells to migrate from the neural tube.

Materials and methods

Embryo preparation and DiI/CRSE labeling

Fertilized Ros-7/Ros chicken eggs (Gold Kist Hatchery, Siler City, NC) were incubated at 37 °C and 70% humidity in a forced-draft incubator. CNCC-A, sham-operated, and control embryos were prepared as described previously (Yelbuz et al., 2002). Cyclopamine (10 μL of 40 μM solution, Toronto Research Chemicals, Inc; North York, ON) was administered within 2 h after surgery, as previously described (Hutson et al., 2006).

Phenotypic analysis

Looping and arterial pole analyses were performed as described previously (Hutson et al., 2006).

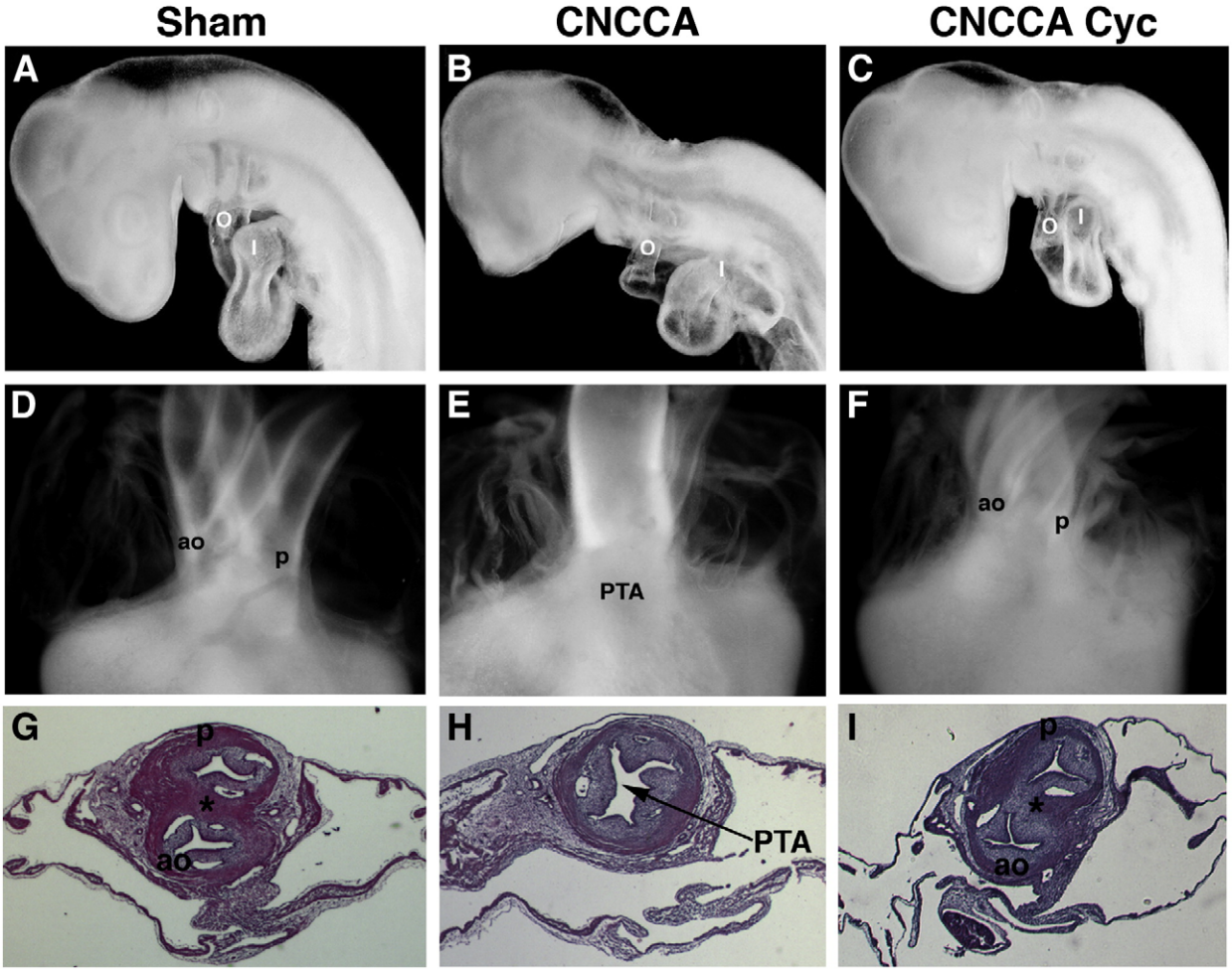

Fig. 1. Reducing hedgehog signaling after CNCC ablation rescues heart looping and outflow septation. (A-C) Heart looping in HH16 chick embryos 24 h after CNCC-ablation. (A) Sham-operated, PBS-treated embryo with a normal heart loop. The inflow limb (I) partially obscures the outflow limb (O). (B) CNCC-A, PBS-treated (CNCC-A) embryo with abnormally looped heart tube. The inflow limb and outflow limb are clearly visible and indicate a shortened heart tube. (C) CNCC-A, Cyc-treated embryo shows somewhat normally looped heart tube. (D) Day 9 heart from sham-operated, PBS-treated embryo with normal outflow alignment and septation. (E) Heart from CNCC-A, PBS-treated embryo with PTA. (F) Heart from CNCC-A, Cyc-treated embryo with a well-divided aorta (ao) and pulmonary trunk (p). (G–H) Transverse sections through the arterial pole of hearts in D–F, respectively. (G) Sham-operated, PBS-treated control heart (from G) has a well-divided outflow. (*) marks the outflow septum. (H) CNCC-A, PBS-treated embryo with an undivided outflow at the level of the semilunar valve and thus has a PTA. (I) CNCC-A, Cyc-treated embryo shows complete division of the arterial trunks. Outflow septum (*).

DiI labeling experiments

Neural tubes of sham-operated and CNCC-A embryos were filled with a mixture of DiI-CM and 5-carboxy-tetramethylrhodamine, succinimidyl ester (CRSE; Invitrogen) as previously described (Waldo et al., 2005) with the following modifications. DiI-CM was dissolved in 100% ethanol at a concentration of 1 mg/ml and further diluted 1:10 in 0.3 M sucrose.

Histology and immunohistochemistry

Embryos were collected at the desired stages and fixed in methacarn for HNK1 and anti-tetramethylrhodamine immunohistochemistry. The embryos were then processed as described previously (Waldo et al., 1998, 1996, 2001). For Pax 7 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) immunohistochemistry, embryos were fixed with 4% paraformaldehyde, saturated in 20% sucrose, embedded in OCT, cryosectioned at 8 μm and visualized with Alexa 488 and rabbit anti-mouse IgG conjugate (Invitrogen).

Results and discussion

Reducing hedgehog signaling in CNCC-A embryos rescues heart looping and arterial pole development

Cyc was applied to sham-operated or CNCC-A HH9–10 chick embryos (Hamburger and Hamilton, 1951) within 2 h of the surgery. As we have previously reported, CNCC ablation caused abnormal looping (Figs. 1A and B), a sign of failure of the secondary heart field to add myocardium to the outflow (Hutson et al., 2006; Yelbuz et al., 2002). Interestingly, heart looping in the CNCC-A embryos treated with Cyc was normal (Fig. 1C; Table S1).

We examined hearts at day 9, a day after arterial pole development is complete. CNCC-ablation resulted in a malaligned common arterial trunk or persistent truncus arteriosus (PTA) in 94% ($n=16$) of the embryos (Figs. 1E, F; Table S2). This indicated that CNCC did not arrive in either the caudal pharynx to modulate FGF8 signaling or in the outflow tract to orchestrate septation. However, 53% of the CNCC-A embryos treated with Cyc appeared by gross examination to have normally aligned aorta and pulmonary trunk (Fig. 1F). This was surprising because CNCC are absolutely required

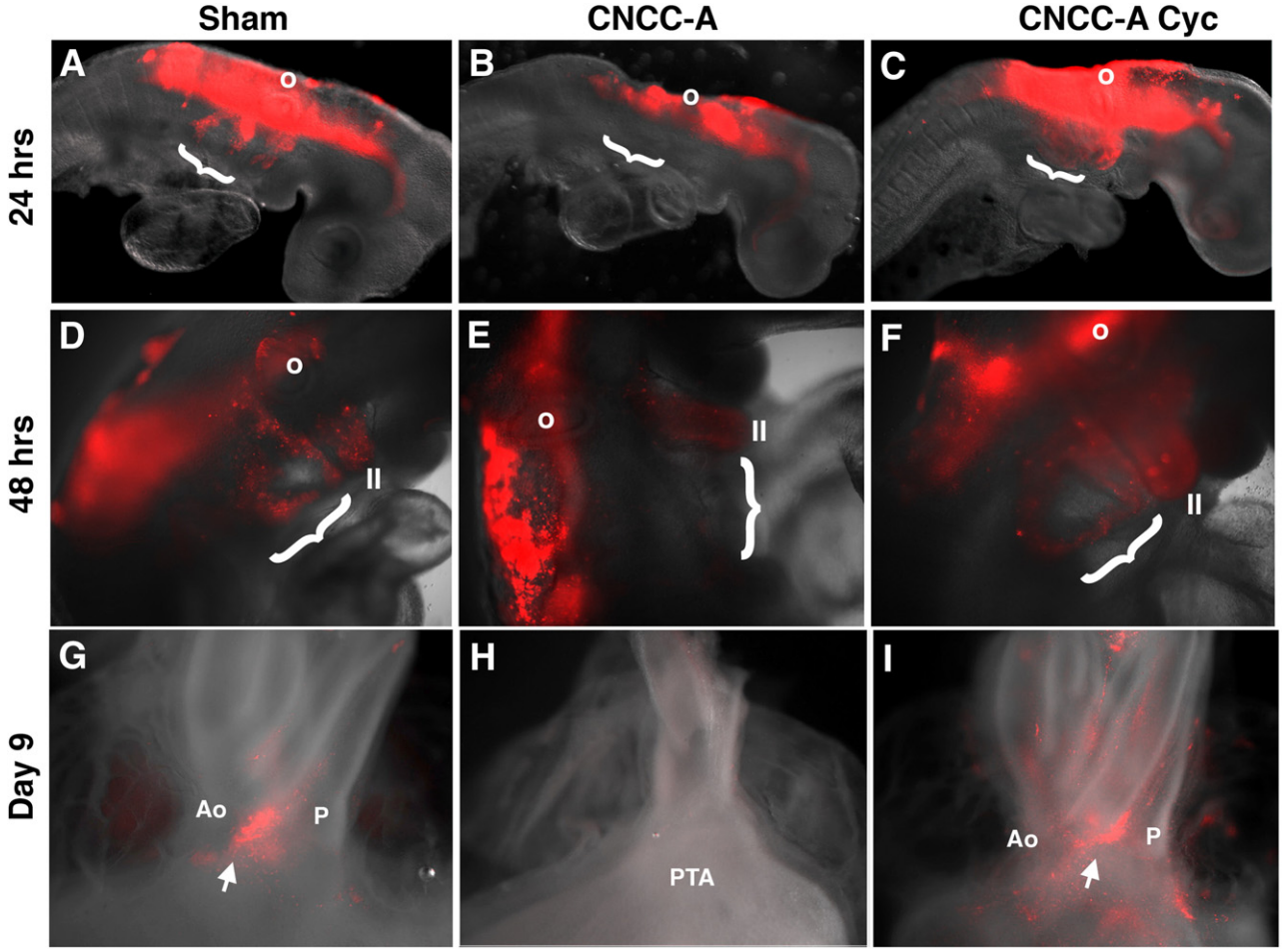

Fig. 2. Neural tube-derived cells migrate through the caudal pharyngeal arches and form the aorticopulmonary septum in CNCC-A embryos treated with Cyc. (A–C) HH15 embryos 24 h after DiI/CRSE (red) labeling cells. (A) Sham-operated, PBS-treated embryo has streams of fluorescent CNCC migrating into the caudal pharyngeal arches (arches III and IV, bracket). Otocyst (o) indicates arch II. (B) CNCC-A, PBS-treated embryo has no fluorescent cells in the pharyngeal arches caudal to arch 2. (C) CNCC-A, Cyc-treated embryo has fluorescent neural tube-derived cells migrating into the caudal pharyngeal arches (Theis et al., 2001). The deformation of the hindbrain region of both the CNCC-A embryos (B and C) indicates the ablated region. (D–F) HH18 embryos 48 h after the neural tube was filled with DiI/CRSE. (D) Sham-operated, PBS-treated embryo showing streams of fluorescent CNCC populating the caudal arches . (E) CNCC-A, PBS-treated embryo showing no fluorescent cells populating the caudal arches . Fluorescent cells can be seen in arch II, which is populated by pre-otic cranial neural crest and not within the CNCC-A region. (F) CNCC-A, Cyc-treated embryo shows abundant fluorescent neural tube-derived cells migrating into the caudal arches . (G–I) Hearts from HH35 (day 9) embryos after DiI/CRSE-labeling. (G) Heart from sham-operated, PBS-treated control embryo showing fluorescent cells in the septum (arrow) between the aorta (Ao) and pulmonary trunk (P). (H) Heart from CNCC-A, PBS-treated embryo has no fluorescent cells in the outflow tract which is not septated (PTA). (I) Heart from CNCC-A, Cyc-treated embryo showing fluorescent cells in the septum between the aorta and pulmonary trunk (arrow).

for septation. In our experience with CNCC ablation, we have never observed less than 95% of CNCC-A animals having hearts with malaligned PTA. To confirm the presence of the outflow septum, we sectioned the hearts. While the septum did not appear completely normal, 8 of 15 hearts from CNCC-A embryos treated with Cyc had a divided aortic and pulmonary outflow at the level of the semilunar valves (Fig. 1I). Most of the CNCC-A embryos treated with Cyc, also had small ventricular septal defects just below the valves (data not shown). These results suggest that reducing hedgehog signaling in an embryo without CNCC can regenerate or recruit cells from the remaining neural tube or neighboring neural crest progenitors to form the outflow septum.

We have recently shown that Shh signaling is required for normal arterial pole development (Dyer and Kirby, 2009). In these studies, we abrogated Shh signaling by treating control embryos with an Shh inhibitor, cyclopamine (Cyc), at stages important for normal arterial pole development (HH14–15) which was 24 h later than the treatment in the current study. Embryos treated with Cyc exhibited pulmonary atresia, pulmonary stenosis, and persistent truncus arteriosus which suggested a CNCC defect. However, we showed that the CNCC were present in the outflow tract but divided the outflow tract into two unequal sized vessels and effectively closed off the pulmonary outlet. The misplacement of the CNCC was due to a reduction in subpulmonary myocardium and not a primary CNCC defectq (Dyer and Kirby, 2009).

After CNCC-ablation, the injured neural tube treated with Cyc generates a new population of migratory cells

Previous studies showed that the neural tube is not able to reconstitute CNCC from cranial to, at the level of, or caudal to the ablated region (Suzuki and Kirby, 1997). To determine if the neural tube was the source of cells that formed the outflow septum in Cyc-treated, CNCC-A embryos, neural tubes were injected with a mixture of DiI-CM and 5-carboxy-tetramethylrhodamine (CRSE) in CNCC-A embryos treated with or without Cyc. The DiI/CRSE labeled the neural tube and any cells emigrating from it. In sham-operated control embryos large numbers of fluorescent cells were observed migrating into the caudal pharyngeal arches 24–48 h after labeling (Figs. 2A, D). Almost no fluorescent cells could be seen in the caudal pharyngeal arches of the CNCC-A embryos (compare bracket Figs. 2A, D with B, E). In about 50% of the CNCC-A embryos treated with Cyc, significant numbers of fluorescent cells were observed in the caudal pharyngeal arches at 24–48 h (Figs. 2C, F). Sham-operated embryos treated with Cyc also showed large numbers of CNCC migrating into the pharyngeal arches (Supplemental Fig. 1). This is similar to the observation made in zebrafish in which initial cranial neural crest migration was not disrupted by Cyc treatment (Tobin et al., 2008).

To determine if the fluorescent cells continued to migrate into the outflow tract, the embryos were allowed to develop. At

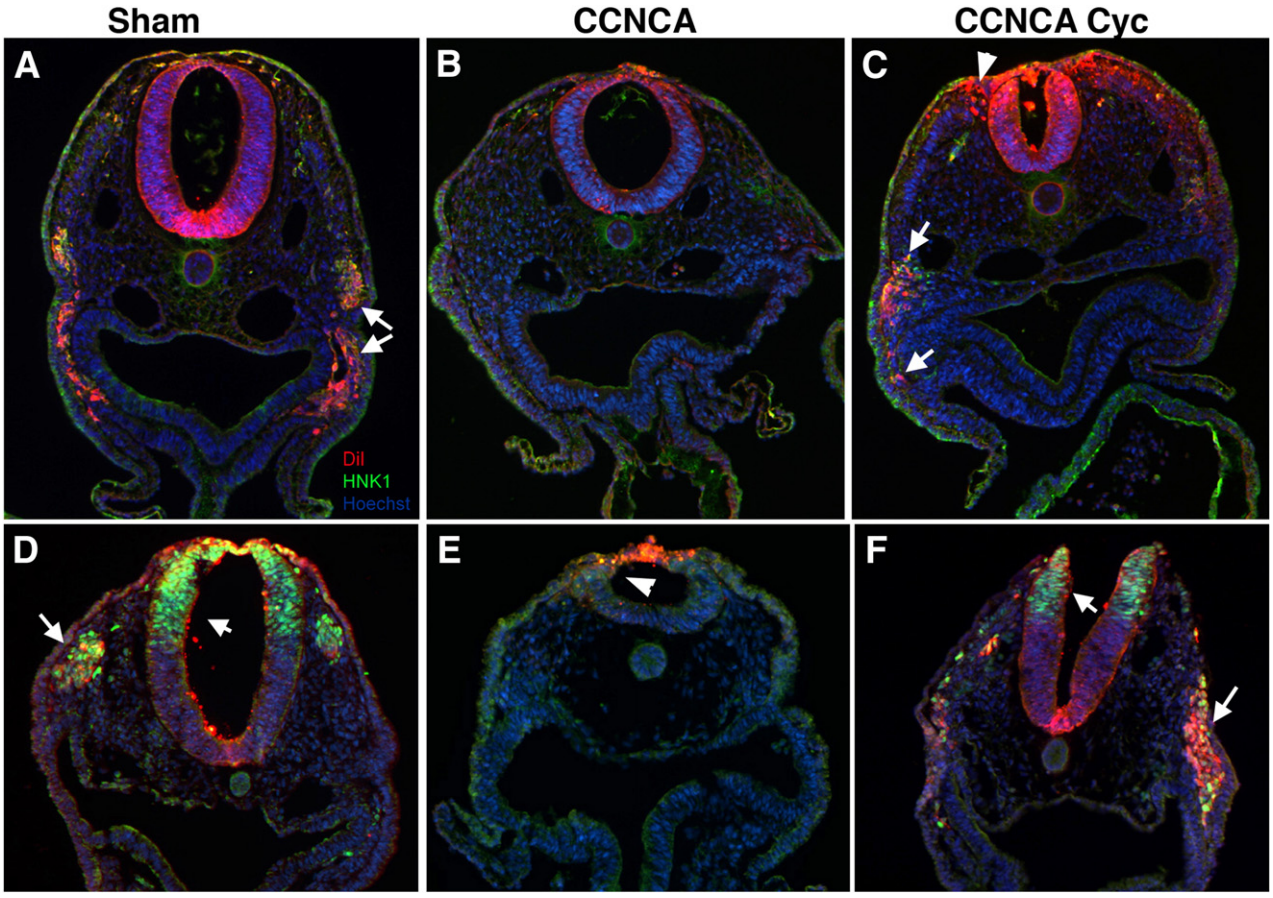

Fig. 3. Cells emigrating from the neural tube in CNCC-A, Cyc-treated embryos express neural crest markers. (A–C) Transverse sections of HH15 embryos injected with DiI/CSRE immunostained with HNK1 antibody (green) and anti-CRSE (red). (A) Sham-operated, PBS-treated embryo shows HNK1 and CRSE co-labeled cells (yellow) near the neural tube and in the pharyngeal arches surrounding the developing arch arteries (arrows). (B) Almost no doubly HNK1-positive and CRSE-positive cells are seen leaving the neural tube or in the pharyngeal region in the CNCC-A, PBS-treated embryo. (C) CNCC-A, Cyc-treated embryo shows HNK1 and CRSE co-labeled cells (yellow) near the neural tube and in the pharyngeal arches (arrows). (D–F) Transverse frozen sections of HH15 embryos immunostained with Pax7 antibody (green) and anti-CRSE (red). (D) Sham-operated, PBS-treated embryo shows Pax7 (arrows) and CRSE co-labeled cells (yellow) near the neural tube and Pax7 expression in the dorsal neural tube (short arrow). (E) CNCC-A, PBS-treated embryo with neither Pax7-nor CRSE-positive cells. The ablated neural tube no longer expresses Pax7 (arrowhead). (F) CNCC-A, Cyc-treated embryo shows Pax7 and CRSE co-labeled cells (yellow, long arrow) near the neural tube and in the pharynx. Pax7 expression is seen in the dorsal neural tube (short arrow). Nuclei are counterstained with Hoechst (blue).

day 9, a wedge of fluorescent CNCC could be seen in the heart from the sham-operated embryos with a clearly divided aorta and pulmonary trunk (Fig. 2G). Fluorescent cells could also be seen scattered along the pharyngeal arch artery-derived great arteries. CNCC form the smooth muscle tunica media of the pharyngeal arch artery-derived great arteries. Hearts from CNCC-A embryos had PTA and showed no fluorescent cells in the common arterial trunk (Fig. 2H). However, hearts from the Cyc-treated, CNCC-A embryos had a large wedge of fluorescent cells separating the aorta and pulmonary trunk, as well as many fluorescent cells scattered along the great vessels (Fig. 2I). Together these results suggest that the outflow septum in CNCC-A, Cyc-treated embryos, is derived from the neural tube in the ablated region.

Migratory cells generated from the injured neural tube follow CCNC migration pathways and express neural crest markers

To further examine the fluorescent cells in the caudal pharyngeal arches, Cyc-treated, CNCC-A embryos were sectioned and immunostained for HNK1 expression and CRSE. In the sham-operated embryos, cells doubly labeled with HNK1 and CRSE invested the developing 4th pharyngeal arch arteries (Fig. 3A). No HNK1 or CRSE-positive cells were seen in the CNCC-A embryo (Fig. 3B). Cells in the CNCC-A embryo treated with Cyc were positive for both HNK1 and CRSE (Fig. 3C). These cells appeared similar in number and distribution to those in sham-operated embryos.

In chicken embryos Pax7 is a marker for neural crest cells and is required for neural crest formation (Basch et al., 2006). It is also

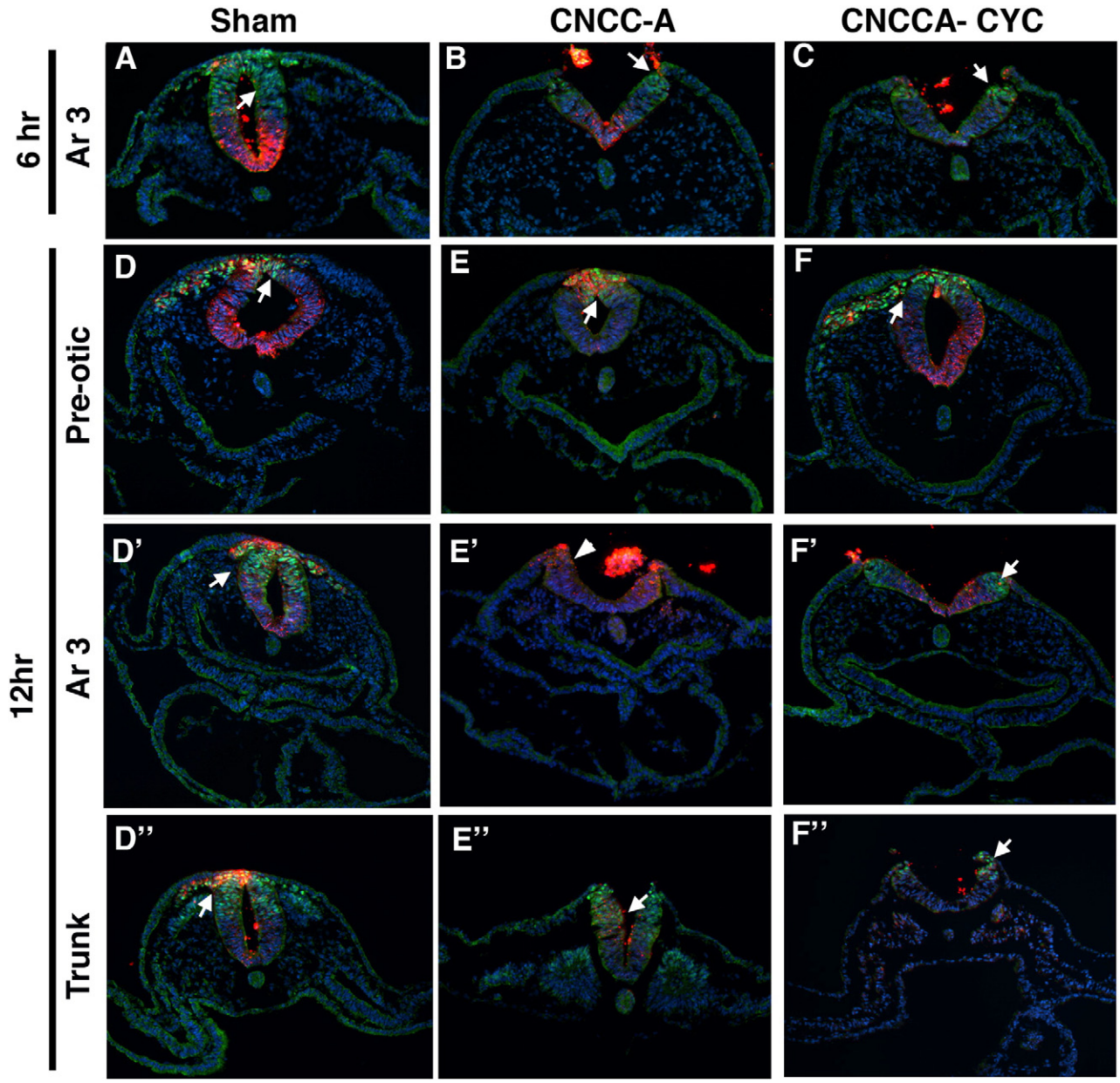

Fig. 4. Cyc treatment maintains Pax7 expression in the dorsal tips of the neural tube in the ablated-region. Pax7 expression (green) and DiI/CRSE labeling (red) in arch 3 region, 6 h (A–C) or 12 h (D″–F″) after various treatments. (A) Pax7 expression is seen in the dorsal neural tube and in newly migrating CNCC in sham-operated, PBS-treated embryo. Pax7 expression is maintained in the dorsal edges of the remaining neural tube of CNCC-A, PBS-treated (B) or CNCC-A, Cyc-treated (C) embryos. (D–D″) Sham-operated, PBS-treated embryo shows Pax7 expression in pre-otic (D), CNCC region (D′) and trunk crest region in the dorsal neural tube and migrating neural crest cells. (E–E″) CNCC-A, PBS-treated embryo shows Pax7 expression in pre-otic (E), and trunk crest regions (E″) in the dorsal neural tube but not in the CNCC region (E′, future arch 3). (F–F″) CNCC-A, Cyc-treated embryo shows Pax7 expression in pre-otic (F), and trunk crest region (F″) in the dorsal neural tube. In contrast to the non-Cyc-treated embryo Pax7 is maintained in the CNCC region (F′).

expressed in the dorsal neural tube and high levels of Shh inhibit Pax7 expression (Briscoe and Ericson, 1999; Ericson et al., 1997a). In sham-operated PBS-treated embryos, Pax7 expression was observed in the dorsal neural tube, in cells migrating from the neural tube, and in cells in the pharyngeal arches 24 h after treatment (Fig 3D). Many of the Pax7-expressing cells were co-labeled with CRSE. In contrast, CNCC-A embryos, Pax7 immunoreactivity was reduced of absent in the neural tube and CRSE labeled cells in the pharyngeal arches (Fig. 3E and Supplemental Fig. 2). However, Cyc-treated, CNCC-A embryos showed Pax7 expression in the dorsal tips of the neural tube, in cells emmigrating from the dorsal neural tube (Supplemental Fig. 3), and cells doubly positive for Pax7 and CRSE were found in the pharyngeal arches (Fig. 3F). As with the HNK1 staining, these cells were located within the normal migration pathway of CNCC. Thus, the new wave of migratory neuroepithelial cells express CNCC markers and follow the normal CNCC migratory pathways.

Patterning of the dorsal–ventral axis of the neural tube depends on counteracting gradients of long-range signals. Wnt and BMP signals promote dorsal identities, while Shh signaling induces ventral identities. Shh and BMPs are secreted signaling factors, acting long-range in a concentration-dependent manner, and antagonize each other during dorsal–ventral fate specification (Liem et al., 2000, 1995). The neural cell phenotype is determined by the differential or modulated expression of homeodomain and bHLH transcription factors in response to activation by BMP and Shh (Barth et al., 1999; Briscoe et al., 2002; Briscoe and Ericson, 1999; Ericson et al., 1997a; Lee et al., 1998; Liem et al., 1997, 1995; Roelink et al., 1995; Timmer et al., 2002). Shh signaling induces differentiation of different cell types along the dorsal–ventral axis of the neural tube. Our results suggest that in the damaged neural tube Cyc treatment decreases Shh levels and causes dorsal–ventral repatterning.

Hedgehog inhibition maintains expression of dorsal neural tube markers in the CNCC-A neural tube

To determine if the dorsal–ventral patterning of the neural tube was altered, we stained sham-operated and CNCC-A embryos treated with or without Cyc for Pax2 expression (Supplemental Fig. 2) Pax2 marks a subpopulation of dorsal and ventral interneurons in the mediolateral neural tube (see Briscoe and Ericson, 2001). After Cyc treatment, Pax2 expression in both Sham and CNCC-A embryos was displaced ventrally and closer to the notochord. This suggested an expansion of the dorsal neural tube after Cyc treatment.

To further characterize the disrupted dorsal–ventral patterning of the neural tube in the ablated region after Cyc treatment, embryos were harvested at 6, 12, and 24 h after Cyc treatment and CNCC ablation, and the neural tube was examined for Pax7 expression. In the sham-operated embryos 6 h after PBS treatment, Pax7 was expressed in the dorsal half of the neural tube and CRSE-Pax7 positive CNCC were observed emigrating from the neural tube (Fig. 4A). Six hours after CNCC ablation, the dorsalmost tips of the remaining neural tube expressed Pax7 in the both the PBS-treated and Cyc-treated, CNCC-A embryos (Figs. 4B and C). Because the ablation only removes the neural folds, the residual Pax7 expression observed in the ablated neural tubes at 6 h is likely ventralmost cells in the Pax7 expression domain. No cells were seen leaving the neural tube at 6 h after ablation in either the CNCC-A or the CNCC-A, Cyc-treated embryos. This suggests a delay in the emigration of the cells in the Cyc-treated CNCC-A embryos. In the sham-operated embryos at 12 h after treatment, Pax7 was still expressed in the dorsal half of the neural tube and more CRSE-Pax7-positive cells were seen leaving the neural tube (Figs. 4D–D″). In contrast, Pax7 was no longer expressed in the neural tube of CNCC-A embryos 12 h after the ablation (Fig. 4E′). However, Pax7 expression was maintained in the dorsal tips of the remaining neural tube in the Cyc-treated, CNCC-A embryos (Figs. 4F–F″). Interestingly, Pax7 expression cranial to and caudal to the lesion was maintained in the CNCC-A embryos comparable to controls (Figs. 4E and E″). This suggests that the ventralizing Shh signal in the CCNC-ablated neural tube was unopposed by the dorsalizing BMP signals resulting in the down-regulation of Pax7 expression. However, the Cyc treatment depressed Shh signaling allowing maintenance of the dorsal Pax7 expression and thus a new wave of migratory cells that delaminate from the neural tube and ultimately support normal cardiovascular development.

The dorsal neural tube is specified by BMP secreted by the roof plate and Shh from the notochord and floor plate. Both BMPs and Shh activate or repress transcription factors required for the specification of progenitor domains and the expression of homeotic genes that define distinct neuronal fates with Shh exerting its influence in a concentration-dependent manner (Ericson et al., 1996, 1997b). A close correlation has been shown between changes in the expression of progenitor domains and subsequent differentiation of different cell types in the spinal cord. A study using chick neural explant assays and recombinant Shh protein or Shh-N blocking antibodies examined the timing of motor neuron development (Ericson et al., 1996) found evidence for two distinct stages of Shh induced commitment to a ventral phenotype. Precursor populations that respond to Shh must be "primed" by Shh at an early stage in development. This priming signal acts to "ventralize" a portion of the neural tube which can be visualized by the repression of Pax7 expression. Shh is required for some time to maintain the ventralized state, because explants from embryos exposed to Shh at HH10 reverted to a Pax7-positive state. In contrast, explants removed 12 h later, at HH12, did not re-express Pax7, suggesting that the cells become independent of Shh signaling after a defined period of exposure. We removed the dorsalizing signals by ablation of the dorsal neural tube and concomitantly blocked ventralizing signals with Cyc during a period in which the dorsal–ventral axis is still not independent of these signals. This allowed generation of a new wave of emigrating CNCC from the damaged region of the neural tube.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.09.013.

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